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Catabolite Inactivation of Wild-type and Mutant Maltose Transport Proteins in *Saccharomyces cerevisiae**

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The maltose transporter of *Saccharomyces cerevisiae* is subject to rapid, irreversible inactivation in the presence of glucose. Loss of transport function was paralleled by a decrease in amount of transporter protein and most likely involves endocytosis and degradation of the protein in the vacuole. This (catabolite) inactivation of Mal61p was triggered not only by glucose but also by 2-deoxy-D-glucose, which cannot be metabolized beyond 2-deoxy-D-glucose phosphate. The signal that targets membrane proteins specifically for catabolite inactivation is unknown. To investigate whether or not specific modification of Mal61p triggers the inactivation, putative protein kinase A and C phosphorylation sites were removed, and the transport activities and levels of the mutant proteins upon addition of glucose were followed in time. Three Mal61p mutants, *i.e.* S295A, T363A, and S487A, exhibited significantly reduced rates of inactivation in the presence of glucose. Likewise, in wild-type Mal61p the rate of inactivation and degradation of the protein paralleled each other in the case of T363A. On the contrary, for the S295A and S487A mutants the rates of protein degradation were slowed down more profoundly than was the loss of transport activity. These observations indicate that (i) some form of modification (*e.g.* phosphorylation) of the protein precedes breakdown, (ii) the modification inactivates Mal61p, and (iii) the inactivation of Mal61p is not necessarily followed by proteolytic degradation.

The first step in maltose metabolism in yeast is performed by the maltose transport protein, which catalyzes the uptake of maltose in symport with one proton. Subsequently, 1,4-α-glucosidase (maltase) hydrolyzes internalized maltose into two molecules of glucose. Maltose fermenting strains of *Saccharomyces cerevisiae* have one or more *MAL* loci. Each locus comprises at least three genes, *MALX1* encodes the maltose transport protein, *MALX2* encodes the maltase, and *MALX3* encodes an activator of *MALX1* and *MALX2* (*X* denotes one of five *MAL* loci, with $X = 1, 2, 3, 4,$ or 6) (1). *MAL* gene expression in maltose-fermenting "wild-type" strains is inducible and glucose-repressed. The latter phenomenon is mediated by the main glucose repression/derepression pathway, which constitutes the transcription factor Mig1p, at least one protein kinase (Snf1p), and several other proteins whose functions are unknown (2). While the main glucose repression/derepression pathway inhibits the synthesis of the proteins involved in the first steps of maltose metabolism, the presence of glucose activates a second mechanism involved in the inactivation (also termed catabolite inactivation) of the maltose transporter but not of maltase (3–7). The inactivation seems to be due to targeting of the protein to the vacuole, where it is proteolyzed (4, 5, 8).

If sugar-respiring or -derepressed (grown on nonfermentable carbon sources) cells of *S. cerevisiae* are fed with glucose or other rapidly fermentable sugars such as fructose or sucrose, a number of metabolic changes occur very rapidly, including inhibition of gluconeogenesis (*e.g.* inactivation of fructose-1,6-bisphosphatase and other gluconeogenic enzymes) and stimulation of glycolysis (activation of phosphofructokinase) (for review, see Ref. 2). The RAS-adenylate cyclase pathway has been implicated in this metabolic switch, as the addition of the fermentable sugar (first messenger) causes a rapid, transient increase in the level of the second messenger cAMP, which in turn activates (specific) protein kinases. A role for cAMP-dependent protein kinase A activity in catabolite inactivation of the high affinity glucose and galactose transporters was suggested from studies in mutants with varying kinase activities (9). Although these studies have been disputed more recently (10), it has not been ruled out that phosphorylation of the transport protein forms the trigger for the controlled degradation of the protein. In this work, we have analyzed catabolite inactivation of Mal61p by mutating putative protein kinase A and C phosphorylation sites. We show that three putative phosphorylation sites, Ser-295, Thr-363, and Ser-487, play a role in catabolite inactivation of the maltose transporter. These comprise both putative protein kinase A and C sites. We also show that inactivation of the transport system and proteolytic degradation can be uncoupled.

MATERIALS AND METHODS

Strains and Growth Conditions—S. cerevisiae 6001B (*MAT***a**, *SUC1, MAL11, MAL12, MAL13, -ura3-52, leu2-3,112*) and 6001BΔ11 (6001B, *mal11Δ::URA3*) (11) were gifts from Dr. C. A. Michels (Queens College, New York). *S. cerevisiae* RH144-3D (*MAT***a**, *ura3, his4, leu2, bar1-1*) and RH266-1D (RH144-3D, *end3*) were gifts from H. Riezman (University of Basel, Basel). Strains were grown in batch culture on YP medium (1% (w/v) yeast extract, 2% (w/v) peptone (pH 6.6)), supplemented with 2% (w/v) glucose or 2% (w/v) maltose, or on minimal medium, containing per liter: 0.2 g of $MgSO_4$, 3.0 g of NaH_2PO_4, 0.7 g of K_2HPO_4, 2.5 g of

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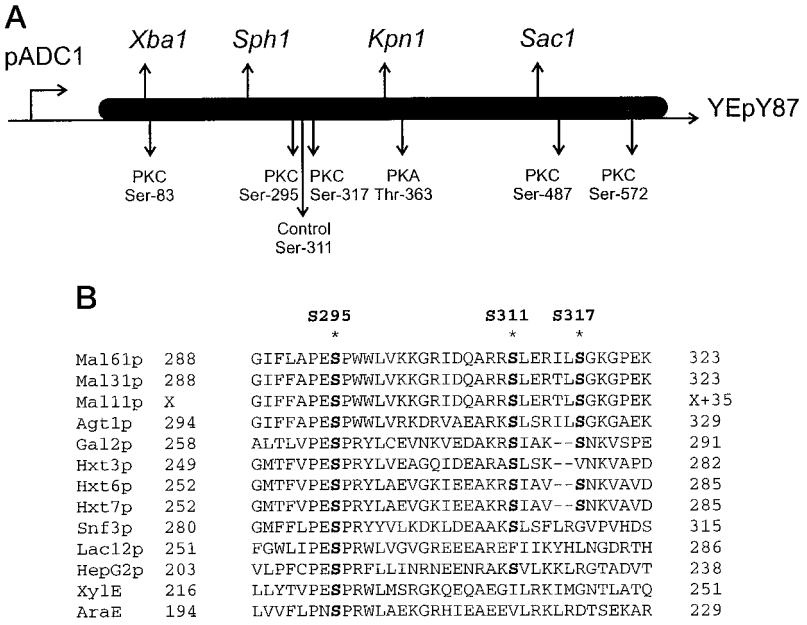

FIG. 1. *A*, maltose transport protein mutants. The 1.85-kilobase pair DNA fragment of YEpY87 containing the *MAL61* gene of *S. cerevisiae* is shown. Restriction sites introduced are indicated in *italics*. The alcohol dehydrogenase promoter is shown as *pADC1*. The *MAL61* gene is depicted as a *thick black bar* and the positions of the phosphorylation sites are indicated. Potential phosphorylation sites are indicated as *PKA* (protein kinase A) or *PKC* (protein kinase C). *B*, multiple sequence alignment of the Mal61p homologs. *Mal61p*, *Mal31p*, *Mal11p*, and *Agt1p* are the maltose transporters of *S. cerevisiae*; *Gal2p* is the *S. cerevisiae* galactose transporter; *Hxt3p*, *Hxt6p*, *Hxt7p*, and *Snf3p* are glucose transporters of *S. cerevisiae*; *Lac12p* is the lactose transporter of *Kluyveromyces lactis*; *HepG2* is the human glucose transporter; and *XylE* and *AraE* are the xylose and arabinose transporters of *E. coli*.

$(NH_4)_2SO_4$ (pH 6.0), 1 ml of Vishniac solution, 1 ml of vitamin solution, 0.5% (w/v) maltose, and when appropriate 1 ml of uracil (40 mg/ml), L-leucine (125 mg/ml), L-histidine (20 mg/ml), or adenine (20 mg/ml). For growth in chemostat culture, the cells were grown in the minimal medium except that K_2HPO_4 was omitted and NaH_2PO_4 was replaced by 1 g of KH_2PO_4. Chemostat cultures were operated at a dilution rate of 0.1 h^{-1}, at 30 °C and pH 5.0 (kept constant by titration with 1 M KOH).

DNA Manipulations—DNA manipulations in *Escherichia coli* were carried out in the strains JM101 (12) or DH5α (13) as described previously (14). Yeast cells were transformed according to the protocol of Gietz *et al.* (15). The plasmid YEpY18 (11) is a yeast multicopy plasmid carrying the *MAL61* gene under control of the *ADC1* promoter. A cassette gene of *MAL61* was constructed using mutagenic primers (see Table I) and YEpY18 DNA as a template for polymerase chain reaction-based synthesis of *MAL61* fragments. Briefly, unique restriction sites were engineered by the polymerase chain reaction 400–500 base pairs apart (see Fig. 1A), which allowed easy subcloning, mutagenesis, and sequencing of the appropriate fragments. The length of the fragment containing *MAL61* in YEpY18 was reduced by digestion of a *Sca*I site 56 bases downstream the TGA stop codon of the *MAL61* gene and a *Sma*I site in the plasmid, thereby reducing the size of the noncoding region by 900 bases. The resulting plasmid YEpY87 was used for mutagenesis of the predicted phosphorylation sites in Mal61p. Site-directed mutagenesis of the phosphorylation sites was carried out by the method of Kunkel *et al.* (16) after subcloning of the appropriate fragments in M13mp18, or, alternatively, by the polymerase chain reaction by using mutagenic oligonucleotides in combination with primers that hybridize at the unique restriction sites. Mutagenic primers are indicated in Table I. All mutations were checked by restriction analysis and DNA sequencing.

Maltase Assay—Maltase activity was measured by following the hydrolysis of *p*-nitrophenyl-α-D-glucoside (*p*NPG)[1] at 400 nm and at 30 °C. The reaction mixture consisted of 1 mM *p*NPG in 100 mM potassium phosphate, pH 6.8. The reaction was started by the addition of cell-free extract to a final concentration of 60–70 μg of protein·ml^{-1}; and stopped by the addition of 2 M Na_2HCO_3. Enzyme activity is expressed as micromoles of *p*NPG converted·min^{-1}·mg of $protein^{-1}$. Cell-free extract was prepared by vortexing 0.1 M potassium phosphate (pH 6.8)-washed cells for 4 min in the presence of glass beads (diameter, 0.23–0.33 mm). Whole cells and debris were removed by centrifugation (5 min; 13,000 × *g*). Protein concentrations were determined in the presence of 0.5% (w/v) SDS using a modified Lowry assay (17).

Inactivation Studies—Cells were harvested from a chemostat culture and resuspended in an equal volume of minimal medium with or without NH_4^+ plus carbohydrate (see "Results") and 12.5 μg/ml cycloheximide to inhibit protein synthesis (18). $(NH_4)_2SO_4$ was replaced by K_2SO_4 when appropriate. The cultures were incubated at 30 °C, and two 1-ml samples were taken every 10 min. One sample was used for immunodetection of Mal61p, and the other sample was used for determination of the initial rate of maltose uptake. The cells were washed once in buffer C (100 mM potassium citrate/PO_4, pH 5.5) and concentrated 20-fold. Transport of maltose in whole cells was measured as described previously (11), at 30 °C using a final [^{14}C-U]maltose concentration of 45 μM.

Immunoblot Analysis—After centrifugation, the cells were resuspended in 200 μl of 50 mM Tris-HCl, pH 7.4, 1% SDS, 8 M urea. Cell-free extract was obtained by vortexing five times, 1 min each, in the presence of glass beads (diameter 0.25–0.5 mm) with 1-min intervals on ice in between. Subsequently, the broken cells were incubated for 10 min at 37 °C, and whole cells and debris were removed by centrifugation (5 min; 6,000 × *g*). Proteins were separated by electrophoresis in 10% SDS-polyacrylamide and transferred to polyvinylidene difluoride (Millipore) sheets by semidry electroblotting (19). Antibodies were raised against a purified amino-terminal 6-histidine tag fusion protein (*N*-$(His)_6$-Mal61p). The antibodies were purified by adsorption against cell-free extract of *S. cerevisiae* 6001BΔ11 (11), and used at serum dilutions of 1:15,000. Primary antibodies were detected with a chemiluminescent detection system using CSPD™ as substrate (Tropix Inc., Bedford, MA). Band intensities were quantified using laser scanning densitometry.

RESULTS

MAL61 Cassette Gene and Mal61p Phosphorylation Mutants—The Mal61 protein of *S. cerevisiae* is phosphorylated *in vivo* (4), but the kinases responsible for this phosphorylation as well as the target sites are unknown. Potential phosphorylation sites located in the cytoplasmic part of the protein were searched for with the program "Prosite" from PC-GENE, and the corresponding residues were replaced by alanines. These residues include putative protein kinase C sites (Ser-295, Ser-317, and Ser-487) as well as a putative cAMP-dependent protein kinase A site (Thr-363) (Fig. 1). As a control the serine residue at position 311, with no predicted modification, was substituted for alanine. To facilitate genetic manipulation of *MAL61*, a cassette gene was constructed, containing unique restriction sites approximately every 400 base pairs (Fig. 1*A*). The mutations creating the new restriction sites were silent (Table I), and the rate of maltose transport and the expression level of the cassette protein were comparable to that of wild-type Mal61p (results not shown). The cassette version of *MAL61* was used for further studies and the construction of putative phosphorylation site mutants.

Catabolite Inactivation of Mal61p—The process of catabolite

[1] The abbreviations used are: *p*NPG, *p*-nitrophenyl-α-D-glucoside; 2-DOG, 2-deoxy-D-glucose.

inactivation is usually triggered by the addition of glucose (3–5). To study catabolite inactivation of the maltose transport protein of *S. cerevisiae* in a genetically well defined background, the strain 6001BΔ11/YEpY87, expressing only Mal61p, was used in most experiments. Cells were grown in a maltose-limited chemostat, which ensures high levels of expression of Mal61p (11). Cells were harvested from the chemostat and at time 0, the cells were shifted to a minimal medium containing glucose and no ammonia. To ensure complete inhibition of protein synthesis, cycloheximide was added to the inactivation medium (18). The activity of Mal61p was estimated from the initial rate of uptake of radioactive maltose, added at a low concentration (45 μM) in order to minimize effects of maltose metabolism. The activity of the maltase was followed by the release of 4-nitrophenol from *p*NPG. Fig. 2 shows that under these conditions the maltase activity decreased less than 10% over a period of 3 h, whereas the maltose transport activity was reduced more than 85% within 1 h. The $t_{½}$ of inactivation of Mal61p-mediated maltose transport activity was 19 min. Maltose transport in the strain 6001B, expressing the *MAL11* gene, inactivates in the presence of glucose with a $t_{½}$ of 40 min (Table II). This indicates variations in catabolite inactivation among naturally occurring homologs of Mal61p. Extracts of 6001BΔ11(YEpY87) cells withdrawn at different time intervals after the medium shift were also analyzed for the presence of the Mal61 protein by immunoblotting. It appeared that loss of maltose uptake activity was paralleled by loss of Mal61p cross-reactivity on immunoblots (Table II). Similar results were obtained with Mal61 protein, which carried an amino-terminal 6-histidine tag (*N*-(His)$_6$-Mal61p). This construct will be described elsewhere.

TABLE I
Mutagenic primers

Restriction site	Position	Mutagenic primer[a]	Amino acid substitution
*Xba*I	210	5′-GACCT**TCTAGA**TGAAGC-3′	Silent
*Spb*I	636	5′-GTGTGG**C**ATGC**C**ATGGG-3′	Silent
*Nar*I	885	5′-GCACCAGAG**GC**G**C**CATGG-3′	S295A
None	885	5′-CCATGG**C**TCCTCTGGTGC-3′	S295E
*Sac*I	993	5′-CGAGGA**GAGCTC**TTGAAAG-3′	S311A
*Hpa*II	951	5′-GAATATTA**GCCGG**TAAAGG-3′	S317A
*Kpn*I	1042	5′-GATGAA**GGTAC**CTACTGGG-3′	Silent
*Mae*I	1086	5′-GGAGAAGA**GCTAGAA**TAGC-3′	T363A
*Hpa*II	1453	5′-G**CCGG**CTTCAAGGC-3′	S487A
*Sac*I	1377	5′-GGTAGTG**GAGCTC**TTC-3′	Silent

[a] Created restriction sites are underlined, mutated bases are printed in bold.

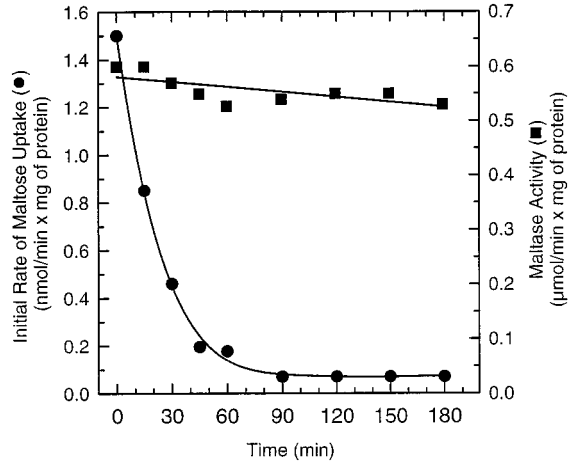

FIG. 2. **Catabolite inactivation of Mal61p and Mal12p.** Strain 6001BΔ11/YEpY87 (wild-type Mal61p), was grown in a maltose-limited chemostat. At time 0, cells were harvested and resuspended in a nitrogen-deficient medium containing 2% glucose plus 12.5 μg/ml cycloheximide. At the times indicated, the cells were harvested and maltose transport (●) and maltase enzyme activity (■) were assayed.

Characterization of Phosphorylation Mutants—All *MAL61* genes with mutations at Ser-295, Ser-311, Ser-317, Thr-363, and Ser-487 as well as the triple mutant S295A,S311A,S317A were able to complement *S. cerevisiae* 6001BΔ11 for growth on maltose, and could be grown in a maltose-limited chemostat. The maltose transport rates catalyzed by the mutated maltose transporters were comparable to the rate catalyzed by wild-type Mal61p (5.3 ± 2.0 nmol maltose·min^{-1}·mg^{-1} of protein). In Fig. 3 catabolite inactivation of Mal61p (wild type) is compared with the inactivation of Mal61p-S295A, Mal61p-S311A, and the triple mutant, Mal61p-S295A,S311A,S317A. The putative phosphorylation site at residue 295 is present in transporters from prokaryotic and eukaryotic species, whereas the sites at 311 and 317 are much less conserved (Fig. 1*B*). Wild-type Mal61p was inactivated significantly faster than the single Mal61p-S295A and the triple Mal61p-S295A,S311A,S317A mutant. The initial rate of maltose uptake of Mal61p decreased to 50% within 20 min, whereas it took 30–35 min for the Ser-295 mutant to reach the same extent of inactivation (Table II). The mutant Mal61p-S311A showed no apparent protection against inactivation and had a $t_{½}$ similar to the wild type. The mutants of Thr-363 and Ser-487 also displayed half-lifes in the presence of glucose that were about twice that of wild-type Mal61p (Table II). The quadruple Mal61p-S295A,S311A,S317A, T363A mutant was not able to complement the 6001BΔ11 strain for growth in a maltose-limited chemostat, although this mutant was able to complement for growth in batch.

Immunoblots showed that in wild-type Mal61p the decrease

TABLE II
Glucose-induced inactivation rate of wild-type and mutant maltose transport proteins

Cells were grown in a maltose-limited chemostat. At time 0, cells were harvested and resuspended in a nitrogen-deficient medium containing 2% glucose plus 12.5 μg/ml cycloheximide, and transport of [^{14}C]maltose was assayed as described under "Materials and Methods"; $t_{1/2}$ values of inactivation were derived from plots of log activity *versus* time (see Fig. 3), and they represent the average ± S.D. for *n* independent experiments.

Strain	Maltose transport protein	$t_{1/2}$ of initial maltose uptake activity	$t_{1/2}$ of Mal61p breakdown
			min
600 1BΔ11/YEpY87	Mal61p	21.8 ± 4.0 (n = 5)	20
6001BΔ11/YEpY107	*N*-(His)$_6$-Mal61p	23.3 ± 1.2 (n = 3)	26.7 ± 3.4 (n = 3)
6001BΔ11/YEpY105	Mal61p-S295A	34.4 ± 4.5 (n = 5)	No decrease during 120 min (n = 2)
6001BΔ11/YEpY114	Mal61p-S295A, S311A, S317A	42	No decrease during 60 min
6001BΔ11/YEpY120	Mal61p-S311A	21	ND[a]
6001BΔ11/YEpY117	Mal61p-S317A	20	ND
6001BΔ11/YEpY130	Mal61p-S295A, S311A, S317A, T363A	No growth in chemostat	ND
6001BΔ11/YEpY125	Mal61p-T363A	44.3 ± 4.9 (n = 3)	48.0 ± 3.0 (n = 2)
6001BΔ11/YEpY140	Mal61p-S487A	41.0 ± 3.0 (n = 2)	65.0 ± 0 (n = 2)
6001B	Mal11p	38.3 ± 0.5 (n = 3)	ND

[a] ND, not determined.

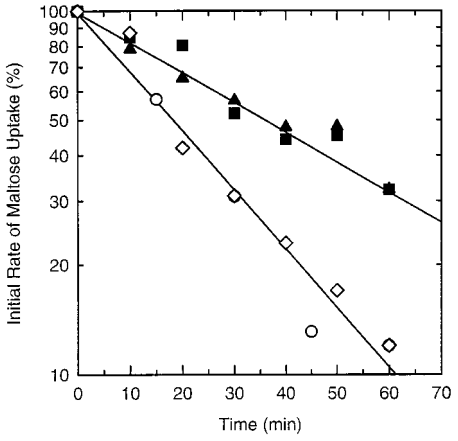

FIG. 3. **Catabolite inactivation of Mal61p phosphorylation mutants.** The inactivation experiment was performed as described in the legend to Fig. 2. The uptake rate at time 0 (5.3 nmol min^{-1} mg^{-1} of protein) was taken as 100%. Strain 6001BΔ11/YEpY87 (wild type) (○), YEpY105 (S295A) (■), YEpY114 (S295A,S311A,S317A) (▲), YEpY120 (S311A) (◇).

TABLE III
Factors affecting catabolite inactivation of Mal61p

Strain 6001BΔ11/YEpY87 cells were grown in a maltose-limited chemostat. At time 0 cells were harvested and resuspended in a medium plus 12.5 μg/ml cycloheximide with or without nitrogen (NH_4^+, as indicated) and containing sugar at the concentration indicated; further details are the same as described in the legend to Table II.

Inactivation medium	$t_{1/2}$ of inactivation min
2% Glucose $-NH_4^+$	21.8 ± 4.0 (n = 5)
2% Glucose $+NH_4^+$	25
2% 2-DOG $-NH_4^+$	23
500 μM Maltose $+NH_4^+$	No inactivation
2% Galactose $-NH_4^+$	No inactivation
2% EtOH $-NH_4^+$	No inactivation (n = 2)

in the level of maltose transport protein occurred at a rate that was similar to the decrease in maltose transport activity (Fig. 4, Table II). This was also true for the T363A mutant. However, the Mal61p-S487A mutant protein disappeared more slowly, the $t_{1/2}$ of disappearance of the protein was 65 min compared to a $t_{1/2}$ of 44 min for the decrease in maltose transport activity. In the S295A and the triple mutant, no significant decrease of the protein was observed. These results show that in the T363A mutant both the decrease in maltose uptake activity and the proteolytic degradation of Mal61p are retarded similarly, whereas in the S295A and S487A mutants the loss of a putative phosphorylation site has a more profound effect on the degradation than on the inactivation.

Since phosphorylation of a serine introduces a negative charge at that position, the effect of phosphorylation might be mimicked by changing the serine to a negatively charged residue. In case of the Ser-295 to glutamate substitution this could result in an increased inactivation rate of Mal61p. Consistent with an enhanced inactivation (degradation) rate of the protein, the Mal61p-S295E mutant was unable to complement 6001BΔ11 for growth on maltose (not shown). However, when this mutant was expressed in a strain with a defect in the *END3* gene, which is necessary for endocytosis, low but significant maltose uptake was observed (Fig. 5). This suggests that in wild-type strains this mutant is subject to rapid "unregulated" endocytosis, which reduces the concentration of Mal61p molecules in the plasma membrane.

Factors Affecting Catabolite Inactivation of Mal61p—To estimate the half-life of the maltose transport protein in the absence of glucose, cells were shifted to a medium containing maltose, galactose, or ethanol instead of glucose. After a shift to

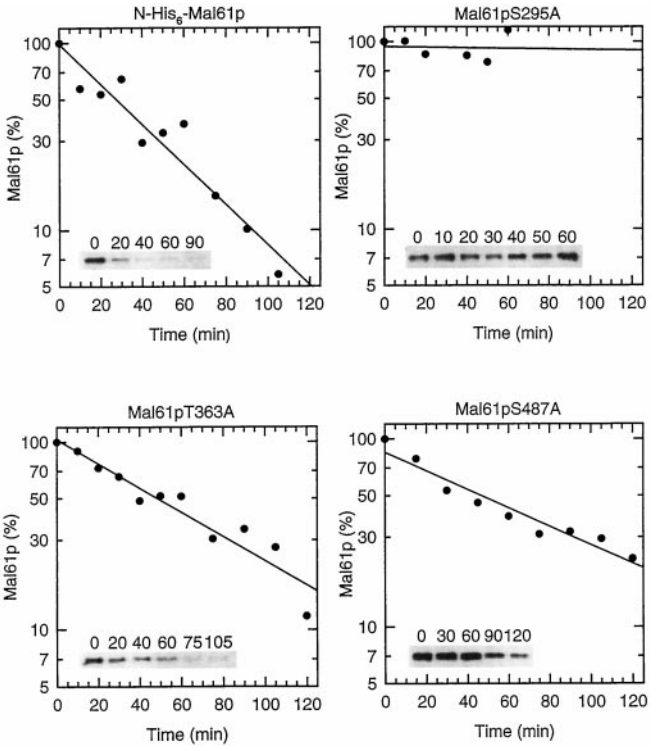

FIG. 4. **Proteolysis of Mal61p.** Total cell extracts were prepared from the same cells as used for the transport assays. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel and analyzed by immunoblotting with antiserum against Mal61p. Each lane contained 25 μg of total protein. Mal61p protein levels were estimated by laser scanning densitometry. The density at t = 0 was taken as 100%.

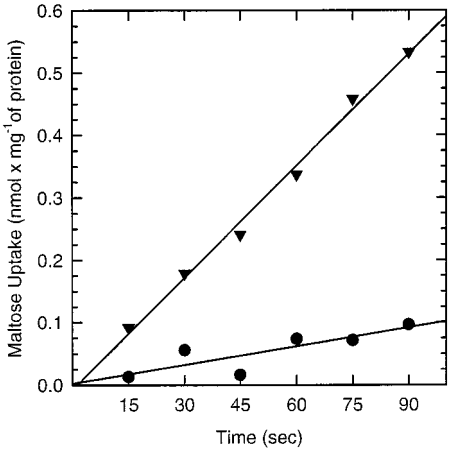

FIG. 5. **Maltose uptake in *S. cerevisiae* endocytosis mutants.** Mal61p-S295E was expressed in *S. cerevisiae* strain RH144–3D (●) and its isogenic strains RH266-1D (*end3*) (▼) using plasmid YEpY151. The cells were grown on mineral medium plus 2% L-lactate and 0.1% yeast extract to an OD at 660 nm of 1.5.

maltose or galactose, the transport activity did not decrease, but actually increased. The maltose uptake activity remained virtually the same with a $t_{1/2}$ >5 h when cells were shifted to medium containing ethanol (Table III, Fig. 6). A shift to a medium containing 2% glucose plus ammonia yielded a $t_{1/2}$ of 25 min (Table III), which indicates that omission of ammonia does not have a significant effect on the inactivation rate. Overall, these results indicate that the intrinsic degradation rate of Mal61p in the absence of glucose is very slow and that inactivation of the protein is specifically triggered by glucose and not by the presence of a fermentable substrate in general. 2-Deoxy-

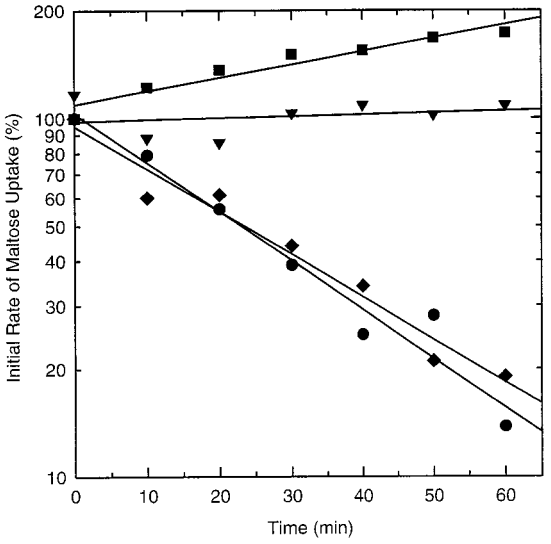

FIG. 6. **Effect of various carbon sources on the inactivation of Mal61p.** The inactivation experiment was performed as described in the legend to Fig. 2 except that different substrates were used in the inactivation medium: 2% glucose (●), 2% ethanol (▼), 500 μM maltose (■), and 2% 2-DOG (♦)

D-glucose (a nonmetabolizable analogue of glucose) was also capable of triggering catabolite inactivation of the maltose transporter (Table III). This suggests that glucose does not have to be metabolized beyond glucose 6-phosphate to trigger catabolite inactivation.

Finally, Medintz *et al.* (4) observed that inactivation of Mal61p was dependent on growth conditions, *i.e.* the protein inactivated more rapidly in nitrogen-starved medium than in rich medium. Since their experiments were carried out with batch-grown cells, whereas ours were done with cells harvested from maltose-limited continuous cultures, we also analyzed the inactivation (decrease in transport activity) of Mal61p and Mal61p-S295A in batch-grown cells. The inactivation rates in batch were reduced to 16 min for wild-type Mal61p and 23 min for the Mal61p-S295A mutant. In the case of Mal11p the effect was even larger with a $t_{1/2}$ of 38 min for cells from a continuous culture and 16 min for batch-grown cells. The expression levels of Mal61p in batch-grown cells were too low to estimate protein degradation accurately (data not shown). Overall these results indicate that the kinetics of inactivation of Mal61p strongly depends on the growth conditions and that the differences in catabolite inactivation between wild-type and mutant proteins are larger in slow growing cells in the chemostat ($D = 0.1\ h^{-1}$) than in exponentially growing batch cells ($\mu_{max} \approx 0.3\ h^{-1}$).

DISCUSSION

The maltose transport protein belongs to a family of proteins from prokaryotic as well as eukaryotic origin, of which the members transport different sugars, *i.e.* MalX1p and Agt1p, maltose; HxtXp and HepG2, glucose; Gal2p, galactose; Lac12p, lactose; XylE, xylose; and AraE, arabinose (Fig. 1*B*) (20). Fig. 1*B* depicts the sequence comparison of the large cytoplasmic loop between putative transmembrane helices VI and VII found in members of this family. This region contains three of the four predicted phosphorylation sites studied here, *i.e.* Ser-295, Ser-317, and Thr-363. We show that substitution of Ser-295, Thr-363, or Ser-487 for alanine has a dramatic effect on glucose-induced inactivation of the maltose transport protein. The triple-mutant Mal61p-S295A,S311A,S317A has the same inactivation time as the single S295A mutant. The T363A mutation caused a similar increase in the half-life of inactivation and Mal61 protein stability, increasing to about twice that of wild-type Mal61p. The S295A and S487A mutations also doubled the inactivation rate, but in these mutants the decrease in protein levels was affected more strongly. The half-life of Mal61p-S487A was about three times that of wild-type Mal61p, whereas no significant decrease in Mal61p-S295A was observed. This finding indicates that inactivation and degradation of Mal61p can be uncoupled and that some form of modification (*e.g.* phosphorylation) of the protein precedes the breakdown. Mal11p has an inactivation rate in the presence of glucose of 38 min, which is comparable to the inactivation rate of the Mal61p-S295A, Mal61p-T363A and Mal61p-S487A mutants. Partial sequencing of the *MAL11* gene revealed that Ser-295, Thr-363, and Ser-487 are present in Mal11p, and the increased inactivation time of Mal11p must therefore be caused by other differences in primary sequence of Mal11p and Mal61p. The overall identity between the two proteins is about 95%.[2]

Catabolite inactivation is not only induced by glucose but also by 2-deoxy-D-glucose (2-DOG). This compound is transported by the glucose transport systems and is readily phosphorylated by yeast hexokinases into 2-DOG-6-phosphate, but it is not metabolized further (21). The catabolite inactivation triggered by 2-DOG is reminiscent to the activation of the RAS-cAMP pathway for which metabolism of glucose beyond glucose 6-phosphate is not needed for induction of the cAMP signal (2). Alternatively, it is also possible that transport of glucose is not required for catabolite inactivation and that external glucose or 2-DOG is sensed by specific receptors in the plasma membrane such as Snf3 or Rgt2 proteins as is the case for glucose repression (22–24).

When cells are shifted to a medium containing galactose, maltose or ethanol instead of glucose, maltose transport activity does not decrease but, in the case of maltose and galactose, increases somewhat. Since protein synthesis is completely inhibited in these cells,[2] the increase in the presence of maltose and galactose must reflect an increase in specific activity of the transporter protein. The steady state maltose concentration in the chemostat was approximately 100 μM,[3] and a switch to a medium containing a higher concentration of sugar (galactose or maltose) may enable the cells to generate more metabolic energy in the form of ATP as well as a higher proton motive force. This higher proton motive force could be the reason for the increase in transport activity. The concentration of maltose in the inactivation medium was deliberately kept low (at 500 μM) in order to avoid substrate induced lysis, as a result of unbridled uptake of maltose (25), but it was higher than the steady state maltose concentration in the chemostat.

In addition to Ser-295, Ser-317, and Ser-487, there are two other protein kinase C phosphorylation sites predicted in Mal61p. One in the N terminus at position 83 and one in the C terminus at position 572 (Fig. 1*A*). These sites have been removed by an internal deletion at the N terminus and by deletion of the C terminus from amino acid 572. Although plasmids carrying these mutations are able to partially complement the *S. cerevisiae* strain 6001BΔ11 for growth on maltose in batch, it was not possible to propagate these strains in the chemostat using conditions described for the wild type (results not shown). The S295E mutant was made in order to introduce an acidic residue (negative charge) at a putative phosphorylation site relevant for catabolite inactivation. We speculate that continuous nonregulated inactivation of the S295E mutant forms the major cause for the inability of the cells to grow on maltose. This is supported by the observation that significant maltose

[2] T. H. C. Brondijk, unpublished results.
[3] M. E. van der Rest, unpublished results.

uptake activity was measured when the Mal61p-S295E mutant was expressed in a strain defective in endocytosis (26).

We showed that mutation of both putative protein kinase C (Ser-295 and Ser-487) and protein kinase A (Thr-363) phosphorylation sites have an effect on catabolite inactivation of the maltose transporter. It should be stressed, however, that protein kinase A and C recognition sites cannot unequivocally be assigned (in fact they may overlap) (27), which leaves quite some room for speculation about the kinase involved in catabolite inactivation. Glucose addition to glucose-starved cells mediates not only a transient increase in cAMP, thereby activating protein kinase A (RAS-cAMP pathway), but it also leads to an increase of the intracellular Ca^{2+} levels (28, 29). Since protein kinase C is stimulated by Ca^{2+}, the intracellular concentration of this inorganic cation could mediate catabolite inactivation as well.

It is possible that, in analogy with the Ste2p G protein-coupled plasma membrane receptor, phosphorylation of the maltose transport protein at one or more residues precedes subsequent ubiquitination of the protein (30). Ubiquitination of the target protein is suggested to function as the endocytosis signal for Ste2p (30). Involvement of ubiquitination in the catabolite inactivation of maltose transport has recently also been suggested (8). One could speculate that phosphorylation of Mal61p at specific sites alters the local structure through which those regions become accessible for ubiquitination.

Catabolite inactivation of the maltose transporter in yeast was also described in recent publications by Riballo *et al.* (5), Medintz *et al.* (4), and Lucero and Lagunas (8). The kinetics of catabolite inactivation reported by Riballo *et al.* (5) is quite different from that described here and also differs from the data of Medintz *et al.* (4). Not only the half-life of inactivation is different (60 min *versus* 20 min in our study) but also the response to glucose is delayed up to 1 h in some experiments (5). The cause of this lag phase is not understood, but could be related to the use of less well defined *S. cerevisiae* strains and/or protein synthesis in the first hour of inactivation. The Lagunas group (8) has also observed that the kinetics of inactivation varies among strains, which makes it difficult to compare the data on catabolite inactivation. Moreover, the strains used by Riballo *et al.*, carry the multicopy plasmid pRM1-1, which specifies the *MAL1* locus, and the cells are grown in batch culture (5). We have observed that the half-life of Mal11p activity in the presence of glucose is longer than that of Mal61p (Table II), whereas the growth conditions (batch *versus* chemostat) strongly affect the kinetics of catabolite inactivation of maltose transport. Medintz *et al.* (4) reported catabolite inactivation of maltose transport with two kinetic components, *i.e.* one with a half-life of about 20 min and one with a $t_{1/2}$ of about 1 h (in nitrogen starvation medium); only the latter is claimed to be associated with degradation of the protein. These authors interpreted the rapid inactivation as the result of a modification (phosphorylation) of the transporter. Our studies confirm this notion as the loss of transport protein is slower than the loss of transporter activity in both Mal61p-S487A and Mal61p-S295A.

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